Pharmaceutical Compositions and Methods for Treating Multidrug Resistant Cancer

Field of the Invention

The invention relates to pharmaceutical compositions and methods for chemotherapy. The invention reverses multidrug resistance to chemotheraputic agents and prevents cardiac damage caused by chemotheraputic agents.

Background of the Invention

Intrinsic or acquired resistance to chemotherapeutic agents is a major contributing factor to failure in cancer treatment. Clinical drug resistance often presents as a multi-drug resistance (MDR) phenotype, characterized as *de novo* resistance to a variety of structurally diverse cytotoxic drugs or as developed cross-resistance to chemotherapeutic agents that have never been used in previous chemotherapy [17]. Although the cellular basis underlying drug resistance is not fully understood, several factors have been identified that contribute to its development. These include drug efflux mechanisms, increased drug inactivation (e.g. glutathione-S-transferase and resistance to alkylating agents), drug target mutation (topoisomerase mutation), altered DNA repair and resistance to apoptosis (p53 mutation, bcl-2 overexpression etc.) [1]. Clinical drug resistance may be caused by any one or a combination of these mechanisms. Increased transmembrane efflux of xenobiotics is one of the best characterized mechanisms of MDR and is known to be mediated through over-expression of adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily members such as P-glycoprotein (P-gp / MDR1), multidrug resistance associated protein (MRP1), or breast cancer

resistance protein (BCRP) [5, 14, 19, 20]. P-gp, the most extensively studied of these transporters, is encoded by the mdr1 gene and found to be overexpressed in many tumor cells, including a variety of leukemias and solid tumors [30]. P-gp over-expression provides protection against a number of chemotheraeutic agents including anthracyclines, vinca alkaloids, antharacenes, camptothecin derivatives, epipodophyllotoxins, and tubulin polymerizing drugs [31].Transfection of the mdr1 gene to drug-sensitive cell lines can transfer the MDR phenotype [28]. In about 30-40% of primary and more than 50% of metastatic breast cancer patient samples, P-gp was overexpressed [16, 27]. Increased expression of P-gp correlates with adverse prognosis and is associated with poor chemotherapy response and overall survival [27].

The prognostic importance of P-gp overexpression shows that the ability to prevent or reverse multi-drug resistance would be clinically valuable. This has led to the identification of a wide variety of compounds that are capable of reversing MDR through the inhibition of P-gp. Preclinical *in vitro* and *in vivo* studies in mice using MDR reversing agents such as verapamil, quinidine and cyclosporine A have demonstrated enhanced anti-MDR tumor activity [9]. To date, clinical trials have been conducted to evaluate the efficacy of MDR reversing agents with mixed results. In some cases, serum levels of reversing agents needed to block P-gp could not be achieved. In other cases, P-gp could be blocked but the levels of chemotherapeutic drugs had to be reduced in order to prevent excessive toxicity. However, some small scale studies in P-gp positive AML and VAD-refractory multiple myeloma showed that incorporation of verapamil or cyclosporin in chemotherapy significantly improved overall survival [6, 18, 26]. New, more potent P-gp inhibitors such as PSC388, GF120918, dexverapamil and XR9576 are

also currently being evaluated in clinical trials and to date, preliminary results indicate that at minimum, it is possible to obtain serum levels of reversing agents sufficient to block P-gp [22, 24]. However, there remains a need for compositions that more effectively prevent multi-drug resistance. As mdr1/P-gp is also expressed in certain normal tissues, blockade of P-gp *in vivo* by reversal drugs inevitably changes drug distribution and metabolism, thus altering the pharmacokinetics of chemotherapeutic agents. As a result, increased accumulation of the drugs in plasma or tissue can cause increased toxicity (Table 1).

TABLE I: Cardiotoxicity of chemotherapy

Drug	Toxic dose range[a]	Toxicity
Amsacrine	Conventional dose	Ventricular arrhythmia
Busulfan	Oral daily dose	Endocardial fibrosis
Cisplatin	Conventional dose	Acute myocardial ischemia
Cyclophosphamide	> 100-120 mg/kg over 2 d	Congestive heart failure, hemorrhagic myocarditis/ pericarditis/necrosis
Daunorubicin	> 550 mg/m^2 (total dose)	Same toxicity as doxorubicin
Doxorubicin	> 550 mg/m^2 (total dose)	Congestive heart failure (cumulative toxic effect), arrhythmias
	< 550 mg/m^2 (total dose)	Cardiac toxicity in the presense of additional risk factors
Fluorouracil	Conventional dose	Angina/myocardial infarction
Interferons	Conventional dose	Exacerbation of underlying cardiac disease
Interleukin-2	Conventional dose	Acute myocardial injury, ventricular arrhythmia, hypotension
Mitomycin	Conventional dose	Myocardial damage similar to radiation-induced injury
Mitoxantrone	> 160-140 mg/m^2 (total dose)	Congestive heart failure, decreased left-ventricular ejection fraction
Paclitaxel	Conventional dose	Bradycardia
Vinblastine	Conventional dose	Myocardial infarction
Vincristine	Conventional dose	Myocardial infarction

[a] Route of administration is IV unless otherwise indicated. Conventional dose is commonly accepted therapeutic range.

Adapted, with permission, from Grover MR, Grieshaber CK: Toxicology by organ system, in Holland JF et al (eds): Cancer Medicine, 4th ed, p 897, Baltimore, Williams & Wilkins, 1997.

Doxorubicin, one of the most potent chemotherapeutic agents for treating hematological malignancies and solid tumors, has dose-limiting cardiotoxicity both in animal models and in cancer patients. In one study, coadministration of cyclosporin and doxorubicin resulted in 55% and 350% increase of area-under-the-curve (AUC) of doxorubicin and its metabolite doxorubicinol respectively (Bartlett, 1994). PSC388, when used in combination with doxorubicin, increased doxorubicin AUC by 10 –fold [12]. Using a murine model, Sridhar showed that the combination of verapamil and doxorubicin increased peak doxorubicin concentration in heart tissue by about 40% compared to doxorubicin alone. This increased tissue doxorubicin level led to severe heart damage and significantly lower survival rate [25]. There is a need for compositions and methods for chemotherapy which do not cause heart damage. The need applies to the most common chemotherapy drugs, such as those shown in Table 2.

Table 2. Cytotoxic drugs which are transported by P-gp.

Anthracyclines	*Vinca* Alkaloids
Doxorubicin	Vincristine
Daunorubicin	Vinblastine
Idarubicin	Vinorelbine
Epirubicin	Others
Epipodophyllotoxins	Mitoxantrone
Etoposide	Dactinomycin
Teniposide	Amsacrine
Taxanes	Trimetrexate
Paclitaxel	Mitomycin
Docetaxel	Mithramycin

Summary of the Invention

The invention relates to a pharmaceutical composition comprising i) ketotifen or an analog thereof and ii) a chemotherapeutic drug subject to multi-drug resistance by P-gp, preferably an anthracycline, more preferably doxorubicin or an analog thereof. The pharmaceutical composition of the present invention is useful for treating cancer. The pharmaceutical composition is also useful for i) circumventing or treating multi-drug resistance in an animal or ii) preventing a chemotherapeutic drug subject to multi-drug resistance by P-gp, preferably anthracycline, more preferably doxorubicin or an analog thereof induced cardiac tissue damage in an animal. The invention also includes kits containing these compositions and methods of use of these pharmaceutical compositions. In variations of the invention, mitoxantrone, VP-16 and vinblastine, or analogs thereof, are useful in the compositions and methods of the invention in place of doxorubicin. Other useful compounds which are subject to P-gp-mediated efflux (preferably those compounds in the same class that have similar activities as doxorubicin, mitoxantrone, VP-16 and vinblastine and which do not cause cause cardiotoxicity) are described below. Ketotifen and its analogs or compounds such as cetirizine and mizolastine are also used with chemotherapy drugs described in this application (or analogs thereof).

The invention relates to a method for treating cancer, comprising treating or incubating cancer cells with a composition that is a Ca^{2+}-mobilizing agonist while concurrently blocking Ca^{2+} influx, whereby the cancer cells are sensitized to cell death induction. Ketotifen is a first generation antihistamine with store-operated Ca^{2+} channel antagonist properties [10]. As a calcium influx blocker, it was previously demonstrated that ketotifen could induce cell death in an activation-enhanced manner in leukemia cells [13], mast cells [23], and breast cancer cells

[29]. In the course of evaluating the ability of ketotifen to induce cell death in breast cancer cells, it was observed that ketotifen could sensitize multi-drug resistant human breast cancer cells to doxorubicin. The invention shows that ketotifen can reverse multi-drug resistance through inhibition of P-gp. More importantly, it shows that ketotifen also reduces cardiotoxicity caused by high dose doxorubicin *in vivo* thus uniquely identifying ketotifen as both a MDR-reversing and cardioprotective agent. Ketotifen restores sensitivity of P-glycoprotein-overexpressing, multi-drug resistant, MCF-7/adr cells to doxorubicin, mitoxantrone, VP-16 and vinblastine. *In vivo*, it was demonstrated that pretreatment of mice with ketotifen caused an increased accumulation of doxorubicin in cardiac tissue, consistent with a block in drug clearance. However, it was also observed that unlike verapamil, ketotifen pre-treatment did not enhance doxorubicin toxicity but in fact provided protection, both at the level of cardiac tissue damage and in survival. The invention provides the surprising invention that ketotifen reverses multi-drug resistance due to P-glycoprotein overexpression and provides cardioprotection to doxorubicin.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Preferred embodiments of the invention will be described in relation to the drawings in which:

Fig.1 Dose response curve of ketotifen and verapamil as MDR reversal compounds. MCF-7/adr cells were treated with different concentrations of reversal compounds with or without 2μM doxorubicin for 24 hours. Cells were harvested , and plated for growth of breast cancer colonies in triplicates. The data are presented as perent of control clonogenicity in the absence of drug ($p< 0.0001$). DXN: doxorubicin.

Fig.2 Influence of 10μM of ketotifen on the toxicities of four cytotoxic drugs. MCF-7/adr cells were treated with different concentrations of chemotherapeutic agents in the presence or absence of 10μM ketotifen for 24 hours. Cells were harvested, washed and plated for growth of breast cancer colonies as described in the Materials and Methods. The data are presented as perent of control clonogenicity in the absence of drug ($p<0.0001$). Ke: ketotifen.

Fig.3 Ketotifen fails to reverse MDR of MCF-7/mx and MCF-7/vp cell lines. MCF-7/mx and MCF-7/vp cell lines were treated and assayed as described in Figs. 1 and 2. The toxicities of mitoxantrone and Vp-16 were evaluated respectively in MCF-7/mx and MCF-7/vp cells. Ke: ketotifen.

Fig.4 Flow Cytometric analysis of intracellular doxorubicin retention. MCF-7/adr cells ($5x10^5$/ml) were incubated with 2μg/ml of doxorubicin at 37°C for 2.5 hours in the presence of different concentrations of ketotifen or verapamil. Cells were washed and resuspended in ice-cold PBS. Doxorubicin relative fluorescence

was measured by flow cytometry. A: 0 μM, b: 2 μM, c: 10 μM ketotifen or verapamil.

Fig.5 Doxorubicin accumulation in heart tissue. Mice were treated with i.p. injection of reveral agents ketotifen or verapamil, followed 30 minutes later by 15mg/kg doxorubicin or saline (as control). Three hours following injection of doxorubicin, three mice were sacrificed in each group and the hearts were excised, rinsed,minced and homogenated. The tissue doxorubicin was extractd with ice-cold acid ethanol solution (0.3N HCl in 50% ethanol). The doxorubicin in the supernatants was measured by fluorescence spectrometry. DXN: doxorubicin, VPL: verapamil, Ke: ketotifen. **: $p<0.01$.

Fig.6 Histological evaluation of cardiotoxicity. Mice were injected with reversal compounds ketotifen (25mg/kg) or verapamil (25mg/kg) followed by doxorubicin (15mg/kg). Three mice in each group were sacrificed 4 days post treatment. The hearts were removed and fixed in 10% formalin. At least 3 sections were made and stained with H.E.. Slides were evaluated by light microscopy (original magnification = 400X). In mice receiving doxorubicin alone, some capillary dilation, degeneration and vacuolization can be readily observed. Combination of verapamil with doxorubicin aggravates these manifestations of cardiotoxicities especially the cytoplasmic vacuolization. Ketotifen, on the other hand, alleviates these pathological changes induced by doxorubicin.

Fig.7 Modulation of doxorubicin toxicity by verapamil or ketotifen. Mice were injected with reversal compounds ketotifen (25mg/kg) or verapamil (25mg/kg) followed by doxorubicin (15mg/kg). Mice were observed for survival for 30 days following treatment. ***: $p<0.001$

Fig. 8 Ketotifen extends survival in mulit-resistant P388/adr murine leukemia cells. Mice were injected with $5x10^5$ p388/adr cells and treated once per week with doxorubicin (4 mg/kg) preceeded by ketotifen (75 mg/kg 30 minutes prior). Both drugs were given intraperitoneal.

Detailed Description of Invention

The inventors have shown that ketotifen can reverse multi-drug resistance through inhibition of P-gp. In addition, the inventors have shown that ketotifen reduces cardiotoxicity caused by high dose doxorubicin in vivo. Thus, the inventors have identified ketotifen as both a MDR-reversing and a cardioprotective agent.

The invention relates to a pharmaceutical composition comprising i) ketotifen or an analog thereof and ii) a chemotherapeutic drug subject to multi-drug resistance by P-gp, preferably an anthracycline, more preferably doxorubicin or an analog thereof. The term subject to mutli-drug resistance means that the chemotherapeutic drug's efficacy is reduced, or may become reduced, in a subject, tissue or cell because of clinical drug resistance associated with multi-drug resistance. A person skilled in the art can assess whether a chemotherapeutic drug is subject to multi-drug resistance by P-gp. For example, the chemotherapeutic drugs to be tested can be incubated with cells, such as P388/adr murine leukemia cells and normal P388 cells as a control. If there is reduced killing of the P388/adr murine leukemia cells as compared to the controls (e.g. normal P388 cells or chemotherapeutic drugs known not to be subject to multi-drug resistance) then the chemotherapeutic agent is subject to multi-drug resistance. In order to determine whether the multi-drug resistance is by P-gp the cells can be stained with a P-gp antibody, such as MRK-16, and the expression of P-gp can be compared to controls. A person skilled in the

art will appreciate that other cell lines could be used, such as comparing the toxicity of the chemotherapeutic agent and P-gp staining in normal MCR-7 cells versus MCF-7/adr cells.

The pharmaceutical composition of the present invention is useful for treating cancer. The pharmaceutical composition is also useful for i) circumventing or treating multi-drug resistance in an animal and ii) preventing a chemotherapeutic drug subject to multi-drug resistance by P-gp, preferably anthracycline, more preferably doxorubicin or an analog thereof induced cardiac tissue damage in an animal. The anthracycline protective effects and reversal of MDR were unknown prior to this invention.

The invention also includes a kit comprising the agents i) ketotifen or an analog thereof and ii) a chemotherapeutic drug subject to multi-drug resistance by P-gp, preferably anthracycline, more preferably doxorubicin or an analog thereof, and directions for administering i) and ii) to an animal, preferably for administering the agents to treat cancer, i) prevent or treating multi-drug resistance in an animal or ii) prevent a chemotherapeutic drug subject to multi-drug resistance by P-gp, preferably anthracycline, more preferably doxorubicin or an analog thereof induced cardiac tissue damage in an animal.

The invention also includes a method for treating cancer in an animal, comprising administering to the animal an effective amount of the pharmaceutical composition of the invention or the agents of the kit of the invention. The cancer can be a solid tumor or a hematological malignancy.

The invention also includes a method for i) circumventing or treating multi-drug resistance in an animal or ii) preventing a chemotherapeutic drug subject to multi-

drug resistance by P-gp, preferably anthracycline, more preferably doxorubicin or an analog thereof induced cardiac tissue damage in an animal, comprising administering to the animal an effective amount of the pharmaceutical composition of the invention.

A preferred embodiment of the invention includes a method for treating cancer in an animal, including

administering to the animal an effective amount of ketotifen or an analog thereof, and

administering to the animal an effective amount of a chemotherapeutic drug subject to multi-drug resistance by P-gp, preferably anthracycline, more preferably doxorubicin or an analog thereof.

Preferably, the ketotifen is administered prior to the doxorubicin, more preferably at least 30 minutes prior to the doxorubicin. In the methods, the ketotifen or analog thereof and doxorubicin or analog thereof are preferably administered orally, intravenously, intraperitoneally, subcutaneously or rectally or by a combination of more than one of the foregoing.

The invention also includes the use of the pharmaceutical compounds and compositions of the invention as a pharmaceutical substance, preferably for treatment of multi-drug resistance.

In addition, the invention includes the use of the pharmaceutical compounds and compositions of the invention for preparation of a medicament, preferably for the treatment of multi-drug resistance.

Cancer

As mentioned above, one embodiment of the invention is a pharmaceutical composition for use in treating cancer. The invention also contemplates methods for treating cancer by administering compounds of the invention (for example, ketotifen and doxorubicin) to an animal. P-gp is found in a variety of leukemias and solid tumors [30]. The term cancer includes any cancer including, without limitation, ovarian cancer, pancreatic cancer, head and neck cancer, squamous cell carcinoma, gastrointestinal cancer, breast cancer (such as carcinoma, ductal, lobular, and nipple), prostate cancer, non small cell lung cancer, Non-Hodgkin's lymphoma, multiple myeloma, leukemia (such as acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, and chronic myelogenous leukemia), brain cancer, neuroblastoma, and sarcomas. [32-36] In a preferred, example the cancer cell overexpresses P-glycoprotein.

Ketotifen

Ketotifen has the chemical name 4-(1-Methyl-4-piperidylidene)-4*H*-benzo[4,5]cyclohepta[1,2-*b*] thiophen-10(9*H*)-one hydrogen fumarate and the molecular formula $C_{23}H_{23}NO_5S$ (Chemical Abstracts Registry Number for Ketotifen is 34580-13-7). It is described, for example, in U.S. Pat. Nos. 3,682,930, 3,749,786, and 5,399,360, and German Patent 2, 111,071. The pharmacology, toxicology, metabolism and the clinical experience with ketotifen has been summarized by Sorkin et al (Focus on Ketotifen. Ed. E.M. Sorkin. In Drugs, September 1990, vol. 40, no. 3, pp. 412-448). Examples of ketotifen analogs are found in U.S. Pat. Nos. 3,682,930 and 3,749,786. The preferred ketotifen analogs are those which are suitable for use in mammals, such as

humans. Methods which may be employed in screening and identifying useful ketotifen analogs and derivatives are described, for example, in U.S. Pat. No. 3,749,786.

Doxorubicin

Doxorubicin has the chemical name 8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-a-*L*-*lyxo*-hexopyranosyl)oxy] -8-glycolyl-7,8,9,10-tetrahydro-6,8,11- trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride. The molecular formula of the drug is $C_{27}H_{29}NO_{11}$•HCl and its analogs are also known in the art. Analogs include mitoxantrone, daunorubicin and N-acetyl daunorubicin. Other doxorubicin analogs are described in US Patent Nos. 4,672,057, 4,345,068, 4,314,054, 4,229,355, 4,216,157, 4,199,571, 4,138,480.

Other compounds are described below. Preferred compounds and analogs have at least 25%, 50%, more preferably at least 75% of the activity of doxorubicin and ketotifen for reversing MDR without cardiotoxicity. Activity may be measured by a MDR assay or cardioprotection study as described in this application.

"Preventing" or "Reversing" drug resistance means inhibiting P-gp to circumvent, reduce or avoid MDR. It does not necessarily mean modifying the cancer cells so that they no longer have the MDR phenotype of overexpressed P-gp.

Pharmaceutical Compositions

The above described substances may be formulated into pharmaceutical compositions for administration to animals in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in

which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Compositions for chemotherapy are described in *Cancer Chemotherapy Handbook* by David S. Fischer, et al. (5th Ed., Mosby-Year Book, Inc. Publication date, May, 1997); *Lippincott's Cancer Chemotherapy Handbook* by Delia C. Baquiran, Jean Gallagher (Lippincott Williams and Wilkins Paperback); Physician's Cancer Chemotherapy Drug Manual, 2002 and CD-ROM by Edward Chu, Vincent T. Devita (2002, Jones & Bartlett Pub)

Administration of a therapeutically active amount of pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

An active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, rectal administration, inhalation, or transdermal application. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered toanimals, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985) or Handbook of Pharmaceutical Additives (compiled by Michael and Irene Ash, Gower Publishing Limited, Aldershot, England (1995)). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and may be contained in buffered solutions with a suitable pH and/or be iso-osmotic with physiological fluids.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the purpose of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

Example 1: Ketotifen specifically reverses MDR mediated by P-pg transporter.

The toxicity of the cytotoxic drugs was measured by clonogenicity assay. As shown in Fig.1A, significant dose-dependent reversal of doxorubicin resistance was observed with ketotifen. Beginning at 1 μM, ketotifen restored doxorubicin toxicity while at 10 μM, the MDR phenotype of MCF-7/adr cells was completely

reversed. Over this concentration range, ketotifen itself is non-toxic to MCF-7/adr cells. The ability of ketotifen to restore sensitivity of MCF-7/adr cells to doxorubicin was compared with verapamil. As shown in Figs. 1A and 1B, both ketotifen and verapamil reverse resistance at similar concentrations. MCF-7/adr cells are also relatively resistant to mitoxantrone, VP-16 and vinblastine. As shown in Fig.2, the sensitivity to these drugs was also restored by 10 μM ketotifen. The IC_{90}s of different cytotoxic drugs were calculated from dose-response curves for MCF-7/adr or MCF-7/wt cells in the presence or absence of 10 mM of ketotifen. As summarized in Table 3, IC_{90} levels on MCF-7/adr cells in the presence of ketotifen are almost identical to those for parental MCF-7 cells. In contrast to its reversing activity on MCF-7/adr cells, ketotifen influenced neither the toxicity of mitoxantrone on MCF-7/mx nor the toxicity of VP-16 on MCF-7/vp cells (Fig.3). These two cell lines exhibit the MDR phenotype by overexpressing BCRP [19] and MRP transporters [21] respectively. Thus, ketotifen is a specific reversing agent for MDR associated with P-gp.

Table 3. IC_{90} (μM) of cytotoxic drugs on MCF-7/wt and MCF-7/adr

	MCF-7/wt	MCF-7/adr	
		-ke	+ke
Doxorubicin	0.12	>10	0.18
Mitoxantrone	0.01	0.22	0.02
VP-16	17	220	20
Vinblastine	0.8	410	1.2

Example 2: Increased intracellular retention of doxorubicin in ketotifen treated MCF-7/adr cells.

Most MDR reversing agents act by inhibiting the transporting activity of P-gp. In order to determine if ketotifen inhibits P-gp activity, the intrinsic fluorescence of doxorubicin was used as a marker and measured drug accumulation by flow cytometry. MCF-7/adr cells pretreated with ketotifen or verapamil were exposed to doxorubicin and fluorescence was measured. As shown in Fig. 4, in the presence of either verapamil or ketotifen, fluorescence from doxorubicin increased in the pre-treated cells. 2 μM of ketotifen increased relative fluorescence by 50%, while 10 mM of ketotifen nearly doubled the fluorescence intensity. This result shows that ketotifen causes an accumulation of doxorubicin in MCF-7/adr cells and that ketotifen mediates its reversal ability through the inhibition of drug efflux.

Example 3: Tissue doxorubicin concentrations in the heart.

To determine the interactions of ketotifen with cytotoxic drugs *in vivo*, mice were given i.p. injections of reversal agent, followed by 15 mg/kg doxorubicin. Tissue concentrations of doxorubicin were determined by measuring doxorubicin fluorescence in heart tissue following different time periods after injection. The 3-hour time point values in different groups were compared as this point was the peak concentration was observed. As observed with verapamil, pre-treatment of mice with ketotifen significantly increased doxorubicin accumulation in the heart in comparison to control (72+/-5 vs 36 +/-3 ng/mg protein, $p<0.01$, Fig.5). This result shows that like verapamil, ketotifen causes a buildup of doxorubicin in tissue, likely due to inhibition of normal drug clearance mechanisms [25].

Example 4: Ketotifen prevents cardiac tissue damage.

Cardiac tissue damage caused by anthracyclines is well known and characterized by cardiac hypertrophy, vacuolization disruption of myofibrils and cell loss [3]. In

order to characterize the effect of combined MDR reversing agent plus doxorubicin treatment on heart tissue, mice were treated with ketotifen or verapamil followed by doxorubicin. Four days later, heart tissue was fixed, sectioned and stained with hematoxylin and eosin. As shown in Fig 6, mice treated with doxorubicin alone demonstrated well known pathological changes including dilation of capillaries, myocyte degeneration and vacuolization in left ventricular tissue (Fig. 6B). Addition of verapamil enhanced cardiac damage caused by doxorubicin (Fig.6C). In contrast, heart tissue from mice pre-treated with ketotifen (25mg/kg) 30 minutes before doxorubicin had observable decreases in the extent of cardiac damage (Fig.6D) with less cell drop-out, maintenance of myofibril structure and less vacuolization.

Example 5: Cardiotoxicity and survival.

Since cardiac damage is reduced in mice receiving ketotifen plus doxorubicin, it was shown that the addition of ketotifen enhances mouse survival. Mice were pre-treated with ketotifen or verapamil, followed by a single treatment with doxorubicin and followed the animals over 5 weeks. Animals were sacrificed when they showed signs of lethargy or distress. As shown in Fig. 7, the survival rate of mice receiving doxorubicin plus verapamil was significantly lowered comparing to those mice treated with doxorubicin alone. For the doxorubicin plus verapamil group, survival rate at day 30 was 0% with median survival time of 12.3 days while 42% of the doxorubicin alone group survived 30 days post treatment with a median survival of 19.3 days ($p<0.001$). In contrast, pre-treatment of mice with ketotifen led to extended survival compared to doxorubicin alone with 57% survival rate at day 30 and a median survival time of 23.2 days ($p<0.001$ compared

to Dox + Verapamil). Since prolongation of survival in mice treated with ketotifen plus doxorubicin correlated with the protection of ketotifen on the cardiotoxicity induced by doxorubicin, these results clearly show that ketotifen enhances survival due to cardioprotection.

In this study, it was shown that the antihistamine ketotifen can reverse multi-drug resistance in MCF-7/adr cells through inhibition of P-gp. This effect is specific in that cells overexpressing BCRP or MRP are not affected by ketotifen. At high concentrations, ketotifen also blocks store-operated Ca^{2+} influx and induces activation enhanced cell death [13, 29]. However, ketotifen's P-gp-inhibitory activity appears to be unrelated to its Ca^{2+} channel blocking activity since the concentrations required for P-gp inhibition are much lower. Furthermore, it was observed that Ca^{2+} ionophores have no effect on the ability of ketotifen to reverse MDR (Zhang and Berger; unpublished), providing additional evidence that ketotifen's MDR reversing activity is unrelated to its Ca^{2+} channel antagonism. It was further shown that pretreatment with ketotifen caused an increased accumulation of doxorubicin in mouse cardiac tissue, consistent with a block in drug clearance. However, unlike verapamil, ketotifen did not enhance doxorubicin toxicity but in fact provided protection, both at the level of cardiac tissue damage and in survival. These observations therefore show that ketotifen is unique in its ability to both reverse multi-drug resistance due to P-glycoprotein overexpression and provide cardioprotection to doxorubicin.

Although the mechanism of cardiotoxicity caused by anthracyclines is not fully understood, it is generally believed that highly active reactive oxygen species (ROS) triggered by anthracycine metabolites may play a central role in the

initiation of a series reactions leading to myocyte damage [11, 15]. While antioxidants have shown some promise as cardioprotective agents *in vitro* and in animal models, clinical trials have not yet provided consistent benefit [4, 8]. Furthermore, the concern arises that the systemic application of antioxidants may also limit the anti-tumor efficacy of doxorubicin. Previous studies investigating the role of mast cell activation products in anthracycline-mediated cardiotoxicity had demonstrated that ketotifen could reduce doxorubicin cardiotoxicity and improve overall survival in a murine model [2]. In another study, doxorubicin was shown to induce mast cell degranulaton and histamine release, consistent with a role for mast cell activation in enhancing cardiac damage [7]. The invention shows the protective effect of ketotifen. Ketotifen's beneficial effect on survival can be partly attributed to cardiac protection based on the observed decrease in severity of cardiac damage in mice pre-treated with ketotifen.

The clinical use of anthracyclines is limited by its cardiotoxicity. Furthermore, schemes employing multi-drug reversing agents typically require reductions in chemotherapeutic dose due to inhibition of drug clearance mechanisms. The observations showing ketotifen as a multi-drug reversing agent with cardioprotective activity shows that this unique combination of properties is clinically useful in the control of multi-drug resistant tumors.

Materials and Methods

Human Breast Cancer Cell Lines and Culture Conditions.

MCF-7 (MCF-7/wt) and its multidrug resistant variant MCF-7/adr cells were used. MCF-7/mx and MCF-7/vp cell lines were also used. Other cell lines could also be used. MCF-7/mx cell line was generated through selection in vitro with

mitoxantrone and overexpresses Breast Cancer Resistance Protein (BCRP) [19]. The MCF-7/vp cell line was selected with etoposide and overexpresses Multidrug Resistance-associated Protein gene (MRP) [21]. All the cell lines were grown routinely as monolayer culture in Dulbecco's Minimal Essential Media (DMEM) supplemented with L-glutamine (2mM), penicillin, streptomycin and 10% heat-inactivated fetal bovine serum (FBS, GIBCO) in an atmosphere of 5% CO_2 at 37°C. The cell lines were passaged weekly.

Chemicals.

Ketotifen, verapamil and all the chemotherapeutic agents (doxorubicin, etoposide VP-16, vinblastine and mitoxantrone) were purchased from Sigma Chemical Co. (St. Louis, MO). Ketotifen was freshly dissolved in DMSO before use, diluted with culture medium and added to the plate at the indicated concentrations. The final concentration of solvent DMSO was always less than 0.1%. All the other drugs were dissolved either in DMSO (VP-16 and vinblastine) or saline and stored at -20°C as stock solutions.

Drug treatments and breast cancer clonogenic assay.

Exponentially growing MCF-7/wt and its three mutants MCF-7/adr, MCF-7/mx and MCF-7/vp were trypsinized, washed with fresh medium and plated in 6-well plates at a density of $1x10^5$/ml. Cytotoxic drugs of different concentrations were applied to cells in the presence or absence of ketotifen or verapamil for 24 hours. Both adherent and non-adherent cells were collected and washed with fresh medium. Cell aliquots ($5x10^3$) were plated in 1ml of 0.3% agar over 1 ml of of 0.5% agar underlayer prepared in IMDM containing 10% horse serum (GIBCO). The upper layer consisted of 20% FBS, 10 μg/ml of bovine insulin, 2.5 mg/ml of

hydrocortisone, $5x10^{-7}$M of 17-b-estradiol (Sigma) and 50 ng/ml of EGF (R & D Systems). Colonies larger than 50 μm in size were scored after 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air.

Flow cytometry.

As doxorubicin itself is a fluorescent substance, the doxorubicin content in MCF-7/adr cells can be measured with flow cytometry. Briefly, cells ($5x10^5$/ml) were incubated with 2 μg/ml of doxorubicin at 37°C for 2.5 hours with or without reversal agents, washed and resuspended in ice-cold PBS. Doxorubicin fluorescence was measured by flow cytometry using a FACStar Plus flow cytometer (FL2, emission at > 570nm,Becton Dickinson). 10^4 cells were analysed for each sample.

Animals and in vivo treatment.

Female Balb/c mice (8-10 weeks of age, 20-22 g of body weight) were purchased from Jackson Laboratory (Maine, USA). Protocols were approved by the Animal Care Committee of the University Health Network. Animals were divided into 6 groups of 15 to 20 mice each and received drug treatments as follows: saline, ketotifen 25mg/kg, verapamil 25mg/kg, doxorubicin 15mg/kg plus saline, doxorubicin 15mg/kg plus ketotifen 25mg/kg, doxorubicin 15mg/kg plus verapamil 25 mg/kg. All the treatments were administrated via i.p. After treatment, mice were kept in sterile environment for six to eight weeks. Acute toxicities and survival was observed for different treatment groups. Mice were sacrificed when they displayed lethargic behaviour or any signs of distress. Three mice from each group were sacrificed on day 4- post treatment. Hearts were removed immediately and fixed in 10% neutral buffered formalin. Tissue sections were made from heart

tissue and stained with hematoxylin and eosin or with 1% Toluidine blue to identify mast cells. All the slides were evaluated by light microscopy for cardiac damage, mast cell density and degranulation.

Doxorubicin concentrations in heart tissues.

3 to 5 mice in each group were treated with the same drug combinations used for survival. Three hours following injection of doxorubicin, mice were sacrificed. Doxorubicin concentrations in heart tissues were determined by flurometric detection of doxorubicin using the method of Sridhar [25]. Briefly, hearts were excised immediately, rinsed with ice cold normal saline, minced with scissors, and homogenized in ice cold ethanol- acid solution (0.3 N HCl in 50% ethanol) using a Polytron homogenizer. The homogenates were centrifuged at 20,000 g for 20 minutes at 4°C. Fluorescence of the supernatants was measured using a Tecan Spectrafluor (excitation wavelenth of 468, emission wavelenth of 590, Hewlett Packard). The doxorubicin standard curve was made by mixing known amounts of doxorubicin with heart tissue and processed using an identical protocol. The fluorescence of supernatant from cardiac tissue without doxorubicin served as background. The concentration of doxorubicin was normalized to total protein content of the same tissue.

Statistical analysis.

All the colony data were analyzed by two-way analysis of variance (ANOVA), with differences between individual means determined by Bonferroni's post-tests. Data were expressed as means ± SEM. The Kaplan-Meier estimate was used to determine differences in the survival periods for mice following different drug combination treatments.

Example 6: Evaluation of the efficacy of ketotifen in enhancing chemotherapy of multidrug resistant leukemia cells

In vitro observations on the ability of ketotifen to reverse MDR, coupled with its ability to partially protect against doxorubicin cardiotoxicity, showed that the combination of ketotifen plus doxorubicin has superior anti-leukemic activity, particularly for multi-drug resistant disease. The combination of ketotifen plus doxorubicin was evaluated in the p388 multi-drug resistant leukemia model. This model has been used in a number of studies to assess efficacy of MDR reversing agents [43-47] and is a useful and rapid approach. Highly multi-drug resistant P388/adr murine leukemia cells were obtained NCI/Frederick Cancer DCT repository. Mice were inoculated with $5x10^5$ p388/adr cells and treated once per week with doxorubicin (4mg/kg) preceded by ketotifen (75mg/kg 30 minutes prior). Both drugs were given intraperitoneal. Ketotifen extended survival 2 days compared to doxorubicin alone ($p=0.02$). See Figure 8. This is comparable to that observed with other drugs such as verapamil [47].

Example 7: Evaluation of the efficacy of ketotifen to reverse MDR and to prevent cardiac damage in a breast cancer model

Human multi-drug resistant breast cancer cells, such as MCR-7/adr cells, are implanted into immune defiant mice, such as female SCID/Rag2m immunodeficient mice, to test the *in vivo* ability of ketotifen to reverse multi-drug resistance, coupled with its ability to protect against doxorubicin cardiotoxicity. The immunodeficiency of these mice prevents them from rejecting human tissue and these mice are therefore suitable hosts for xenographs.

MDR Reversal

On day 1, 1.5 mg Estrogen pellets are implanted at the base of the neck. On day 2, mice are inoculated with 10 million MCF-7/adr cells in a 0.1 ml volume subcutaneously in the flank. When the tumors are 5 mm in diameter, the mice (at least 4 per group) are treated with ketotifen (typically 75 mg/kg intraperitoneally) followed by doxorubicin (typically 4 mg/kg intrperitoneally) 30 minutes later. This treatment is repeated weekly and tumor size and body weight are examined over time.

Cardiac Function

The mice and their normal counterparts are challenged with a single i.p. injection of doxorubicin (15mg/kg). One cohort of 10 mice from each group are evaluated for lethality, as defined by failure to thrive to the point where the animals have reduced mobility and are impaired for feeding. Reduced mobility is defined as decreased mobility such that nesting functions or other normal activity is decreased. Impaired feeding means that the mice are unable or uninterested in consuming solid food or cannot reach up to drink liquid from the suspended water bottle. A second cohort (5 mice per group) is evaluated for cardiac function. To be considered able to protect against cardiotoxicity the results must be statistically significant compared to the controls. Typically, hearts exposed to excessive levels of anthracyclines undergo cardiac hypertophy and cell loss [37,38]. This manifests itself in impaired heart function that can be evaluated by measuring cardiac function non-invasively and invasively as well as through pathological analysis of affected tissue. For non-invasive measurements, echocardiography is used. Echocardiography is a commonly used clinical tool in the management of patients undergoing anthracycline treatment [39,40]. At 4 days post-treatment, anesthetized

mice are placed on a mouse pad equipped with a heater, continuous ECG output and rectal temperature monitoring. Transthoracic 2-dimensional, M-mode and Doppler echocardiographic examination is performed using an Acuson Sequoia C256 system. In addition, treated mice will undergo ex-vivo assessment of cardiac function. The hearts are isolated in a Langendorff preparation with an oversized intraventricular balloon at graduated incremental volumes to derive intrinsic pressure-volume (P-V) relationship, to determine load independent ventricular systolic function; and diastolic compliance P-V relationship to characterize diastolic relaxation [41]. Finally, heart tissue is analysed pathologically for apoptosis by TUNEL assay, fibrillar collagen content is determined using Picosirius Red staining in conjunction with light microscopy and videodensitometry, and collagen content are measured using hydroxyproline assay with Ehrlich's reagent and absorbance at 550nm as previously described [42]. These measurements provide an accurate and quantitative picture of the extent of cardiomyopathy in these mice. The animals pretreated with ketotifen have significantly better heart function and less damage than those animals not pretreated with ketotifen.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication,

patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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